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Award Number: W81XWH-06-1-0553

TITLE: Prolactin Receptor Coupling to Jak-Stat Pathways in Breast Cancer

PRINCIPAL INVESTIGATOR: Lynn Neilson

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, PA 19107

REPORT DATE: August 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2007	Annual Summary	15 May 2006 - 31 Jul 2007

4. TITLE AND SUBTITLE
Prolactin Receptor Coupling to Jak-Stat Pathways in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0553

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Lynn Neilson

E-Mail: lmm36@georgetown.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Thomas Jefferson University
Philadelphia, PA 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Prolactin receptors (PRLR) have been considered selective activators of tyrosine kinase Jak2 but not Jak1, Jak3 or Tyk2. We now report marked PRL-induced tyrosine phosphorylation of Jak1, in addition to Jak2, in a series of human breast cancer cell lines, including T47D, MCF7, and SKBR3. In contrast, PRL did not activate Jak1 in immortalized, non-cancerous breast epithelial lines HC11, MCF10A, ME16C, and HBL-100, or in CWR22Rv1 prostate cancer cells or MDA-MB-231 breast cancer cells. However, introduction of exogenous PRLR into MCF10A, ME16C, or MDA-MB-231 cells reconstituted both PRL-Jak1 and PRL-Jak2 signals. PRL activated Jak1 through a Jak2-dependent mechanism in T47D cells, based on disruption of PRL activation of Jak1 following Jak2 suppression by 1) lentiviral delivery of Jak2 shRNA, 2) adenoviral delivery of dominant-negative Jak2, and 3) AG490 pharmacological inhibition. Finally, suppression of Jak1 by lentiviral delivery of Jak1 shRNA blocked PRL activation of ERK and Stat3, and suppressed PRL activation of Jak2, Stat5a, Stat5b, and Akt, as well as tyrosine phosphorylation of PRLR. The data suggest that PRL activation of Jak1 represents a novel, Jak2-dependent mechanism that may serve as a regulatory switch leading to PRL activation of ERK and Stat3 pathways, while also serving to enhance PRL-induced Stat5a/b and Akt signaling.

15. SUBJECT TERMS
Prolactin, Jak1, breast cancer, signal transduction

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

Prolactin (PRL) signaling through the Jak2-Stat5 pathway has been well characterized and implicated for the normal growth, development, and differentiation of human breast epithelia. PRL has also been suggested to have a breast tumor-promoting effect in humans, and more than 95% of breast cancers express PRL receptors (PRLR). Our lab has discovered that PRL can additionally signal through Jak1 in breast cancer cells but not in normal mammary epithelial cells. The specific aims approved in this training grant explore our initial hypothesis that PRL promotes breast cancer invasiveness through abnormal activation of a novel PRL-Jak1-Stat3 pathway, which is mediated by the interleukin-6 signal transducer, gp130, through a mechanism that requires a membrane proximal region of the PRLR but does not require Jak2 activation.

Body

We are pleased to report the successful completion and publication of our progress in the peer-reviewed journal *Molecular Endocrinology* (see attached).

Many developments in this project have been made throughout the funding period, significantly altering our initial hypothesis. First, Task 1 has been completed, establishing that PRL-induced Jak1 activation is instead *dependent* on Jak2 in T47D breast cancer cells, based on disruption of PRL activation of Jak1 following Jak2 suppression by 1) lentiviral delivery of Jak2 shRNA, 2) adenoviral delivery of dominant-negative Jak2, and 3) AG490 pharmacological inhibition. These data are described fully in the attached article. In addition, PRL activation of Jak1 was explored in Jak2-knockout mouse mammary tumor cells (Task 1C), which is also described in the attached article. Briefly, $Jak2^{fl/fl}$ JDM1.1 cells expressed both endogenous Jak1 and Jak2, while Jak2-deficient ($Jak2^{-/-}$) JDM1.2 cells expressed Jak1 but lacked the Jak2 protein. Regardless of Jak2 expression status, PRL failed to activate either Jak2 or Jak1 in JDM1.1 and JDM1.2 cells. To overcome the problem of low PRLR expression in the JDM cell lines, and to effectively reconstitute PRLR expression and determine whether PRLR would restore PRL activation of both Jak2 and Jak1, cells were infected with adenovirus expressing the long form of the PRLR (hPRLR-L). Interestingly, expression of exogenous hPRLR-L by adenoviral gene delivery restored PRL activation of Jak2 but not Jak1 in JDM1.1 cells. No activation of Jak1 was seen in Jak2-deficient JDM1.2 cells. These observations suggested that PRLR and Jak2 are required but not sufficient for PRL activation of Jak1.

To supplement our finding that PRL activates Jak1 in T47D and MCF7 breast cancer cells but not HC11 near-normal mouse mammary epithelial cells, we extended the study to include additional cell lines: ER-negative SKBR3 and MDA-MB-231 breast cancer cells, CWR22Rv1 prostate cancer cells, and MCF10A, ME16C and HBL-100 immortalized, non-cancerous human mammary epithelial cells. PRL induced marked tyrosine phosphorylation of Jak1, in addition to Jak2, in T47D, MCF7, and SKBR3 breast cancer cells. In contrast, PRL did not activate Jak1 in HC11, MCF10A, ME16C, HBL-100, CWR22Rv1, or MDA-MB-231 cells. However, introduction of exogenous PRLR into MCF10A, ME16C, or MDA-MB-231 cells reconstituted both PRL-Jak1 and PRL-Jak2 signals. These data are described fully in the attached article.

In addition, we examined the biological implication of PRL activation of Jak1 in T47D and SKBR3 breast cancer cells by determining downstream signaling of this pathway. Suppression of Jak1 by lentiviral delivery of Jak1 shRNA blocked PRL activation of ERK and Stat3, and

suppressed PRL activation of Jak2, Stat5a, Stat5b, and Akt, as well as tyrosine phosphorylation of PRLR in T47D and SKBR3 breast cancer cells. The data suggest a role for Jak1 as a broadly acting positive modulator of PRLR-Jak2 signals, with some signals being more dependent than others, which is discussed in the attached article.

Furthermore, experiments outlined in Task 2 have also been underway. We have generated lentivirus to express gp130 shRNA that effectively knocks down protein in T47D and SKBR3 cells, and we are currently employing this tool to determine if PRL activation of Jak1 is dependent on gp130.

Key Research Accomplishments

The following key findings have resulted from this training grant:

- PRL induced marked tyrosine phosphorylation of Jak1, in addition to Jak2, in a series of human breast cancer cell lines, including T47D, MCF7, and SKBR3. In contrast, PRL did not activate Jak1 in immortalized, non-cancerous breast epithelial lines HC11, MCF10A, ME16C, and HBL-100, or in CWR22Rv1 prostate cancer cells or MDA-MB-231 breast cancer cells.
- Introduction of exogenous PRLR into MCF10A, ME16C, or MDA-MB-231 cells reconstituted both PRL-Jak1 and PRL-Jak2 signals.
- Introduction of exogenous PRLR into JDM1.1 cells restored PRL activation of Jak2 but not Jak1.
- PRL activated Jak1 through a Jak2-dependent mechanism in T47D cells, based on disruption of PRL activation of Jak1 following Jak2 suppression by 1) lentiviral delivery of Jak2 shRNA, 2) adenoviral delivery of dominant-negative Jak2, and 3) AG490 pharmacological inhibition.
- Suppression of Jak1 by lentiviral delivery of Jak1 shRNA blocked PRL activation of ERK and Stat3, and suppressed PRL activation of Jak2, Stat5a, Stat5b, and Akt, as well as tyrosine phosphorylation of PRLR in T47D and SKBR3 breast cancer cells.

Reportable Outcomes

Manuscripts:

Neilson LM, Zhu J, Xie J, Malabarba MG, Sakamoto K, Wagner KU, Kirken RA, and Rui H. Coactivation of Jak1 Positively Modulates Prolactin-Jak2 Signaling in Breast Cancer: Recruitment of ERK and Stat3 and Enhancement of Akt and Stat5a/b Pathways. (2007) Molecular Endocrinology 21(9):2218-2232.

Abstracts:

Neilson LM, Zhu J, Xie J, Malabarba MG, Kirken RA, and Rui H. Prolactin activates tyrosine kinase Jak1 in human breast cancer by a Jak2-dependent mechanism. Gordon Research Conference on Prolactin and Growth Hormone Family, Ventura CA, January 29-February 3, 2006. Poster

Neilson LM, Zhu J, Xie J, Malabarba MG, Kirken RA, and Rui H. Prolactin activates tyrosine kinase Jak1 in human breast cancer by a Jak2-dependent mechanism. Nineteenth Annual Georgetown University Student Research Days Competition and Exposition, February 2006. Poster

Neilson LM, Zhu J, Xie J, Malabarba MG, Sakamoto K, Wagner KU, Kirken RA, and Rui H. Prolactin activates tyrosine kinase Jak1 in human breast cancer: Evidence for a Jak2-dependent mechanism. Keystone Symposia on Jaks, Stats, and Immunity, Steamboat Springs CO, January 5-10, 2007. Poster

Conclusion

In summary, we report a new PRLR-Jak2-Jak1 signaling axis based on consistent observations in several human breast cancer cell lines, including ER-positive T47D and MCF7 and ER-negative SKBR3. The data indicate a major role of Jak1 as an enhancer of PRL-Jak2 signals in T47D and SKBR3 cells, where the dependence of PRL activated ERK and Stat3 pathways on Jak1 raises the possibility that Jak1 represents a new and conditional branching point of the PRL receptor signaling network that is active in breast cancer. From a pharmacological perspective, the novel involvement of Jak1 in PRLR signaling, at least in a subset of breast cancer, may represent a new pharmacological target. Specifically, combined inhibition of Jak2 and Jak1 may synergize to suppress growth and survival-promoting PRL effects in some tumors and be advantageous over inhibition of Jak2 alone. Furthermore, if Jak1-specific pathways were to preferentially mediate tumor-promoting effects of PRL, inhibitors of Jak1 may be useful in breast cancer treatment to preferentially disrupt select PRL-induced signals while having less effect on other signaling pathways. Future directions initiated by this project will be to determine the role of Jak1 in PRL biology and signaling in breast cancer, and to further investigate the molecular mechanisms underlying PRL-Jak2 activation of Jak1.

Appendices

Publication: **Neilson LM**, Zhu J, Xie J, Malabarba MG, Sakamoto K, Wagner KU, Kirken RA, and Rui H. Coactivation of Jak1 Positively Modulates Prolactin-Jak2 Signaling in Breast Cancer: Recruitment of ERK and Stat3 and Enhancement of Akt and Stat5a/b Pathways. (2007) Molecular Endocrinology 21(9):2218-2232.
See Following Pages

0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(9):2218–2232

doi: 10.1210/me.2007-0173

Coactivation of Janus Tyrosine Kinase (Jak)1 Positively Modulates Prolactin-Jak2 Signaling in Breast Cancer: Recruitment of ERK and Signal Transducer and Activator of Transcription (Stat)3 and Enhancement of Akt and Stat5a/b Pathways

Lynn M. Neilson, Jianquong Zhu, Jianwu Xie, M. Grazia Malabarba, Kazuhito Sakamoto, Kay-Uwe Wagner, Robert A. Kirken, and Hallgeir Rui

Kimmel Cancer Center (L.M.N., J.Z., J.X., H.R.), Department of Cancer Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; Tumor Biology Graduate Program (L.M.N.), Lombardi Comprehensive Cancer Center, Department of Oncology, Georgetown University Medical Center, Washington, D.C. 20057; IFOM (M.G.M.), the FIRC Institute for Molecular Oncology Foundation, 20139 Milan, Italy; Dipartimento di Medicina (M.G.M.), Chirurgia ed Odontoiatria, Università degli Stadi di Milano, 20122 Milan, Italy; Eppley Institute for Research in Cancer and Allied Diseases and the Department of Pathology and Microbiology (K.S., K.-U.W.), University of Nebraska Medical Center, Omaha, Nebraska 68198; and Department of Biological Sciences (R.A.K.), University of Texas, El Paso, Texas 79968

Prolactin (PRL) receptors (PRLRs) have been considered selective activators of Janus tyrosine kinase (Jak)2 but not Jak1, Jak3, or Tyk2. We now report marked PRL-induced tyrosine phosphorylation of Jak1, in addition to Jak2, in a series of human breast cancer cell lines, including T47D, MCF7, and SKBR3. In contrast, PRL did not activate Jak1 in immortalized, noncancerous breast epithelial lines HC11, MCF10A, ME16C, and HBL-100, or in CWR22Rv1 prostate cancer cells or MDA-MB-231 breast cancer cells. However, introduction of exogenous PRLR into MCF10A, ME16C, or MDA-MB-231 cells reconstituted both PRL-Jak1 and PRL-Jak2 signals. *In vitro* kinase assays verified that PRL stimulated enzymatic activity of Jak1 in T47D cells, and PRL activated Jak1 and Jak2 with indistinguishable time and dose kinetics. Relative Jak2 deficiency did not cause PRLR activation of Jak1, because overexpression of Jak2 did not interfere with PRL activation of Jak1. Instead, PRL activated Jak1 through a Jak2-dependent mechanism, based on disruption of PRL activation of Jak1 after Jak2 suppression by 1) lentiviral delivery of Jak2 short hairpin RNA, 2) adenoviral delivery of dominant-negative Jak2, and 3) AG490 pharmacological inhibition. Finally, suppression of Jak1 by lentiviral delivery of Jak1 short hairpin RNA blocked PRL activation of ERK and signal transducer and activator of transcription (Stat)3 and suppressed PRL activation of Jak2, Stat5a, Stat5b, and Akt, as well as tyrosine phosphorylation of PRLR. The data suggest that PRL activation of Jak1 represents a novel, Jak2-dependent mechanism that may serve as a regulatory switch leading to PRL activation of ERK and Stat3 pathways, while also serving to enhance PRL-induced Stat5a/b and Akt signaling. (*Molecular Endocrinology* 21: 2218–2232, 2007)

PROLACTIN (PRL) is a pituitary hormone required for pregnancy-associated lobuloalveolar development and terminal differentiation of mammary epithelium, and for milk production during lactation (1). PRL binds to specific transmembrane receptors on target cells and is known to activate the receptor-associated Janus tyrosine kinase (Jak)2 but not other members of the Janus kinase family, Jak1, Jak3, or Tyk2 (2–5). Upon PRL-induced receptor aggregation, activated Jak2 in turn phosphorylates cytoplasmic tyrosine residues of the receptor and cytoplasmic mediator proteins such as signal transducer and activator of transcription (Stat)5a, Stat5b and other downstream effectors to perpetuate the PRL signals (6). Consistent with a prerequisite function of Jak2 downstream of PRL receptors (PRLRs), mice with mammary gland-specific knockout of the Jak2 gene (7) display a mammary phenocopy of PRL and PRLR gene knockout mice (8, 9) and are characterized by lack of lobuloalveolar development and lactation. Likewise, mice with

First Published Online June 5, 2007

Abbreviations: DMBA, 7,12-Dimethylbenz(A) anthracene; DN-Jak2, dominant-negative Jak2; EGF, epidermal growth factor; EGFR, EGF receptor; FAK, focal adhesion kinase; Jak, Janus tyrosine kinase; MOI, multiplicity of infection; OSM, oncostatin-M; PRL, prolactin; PRLR, prolactin receptors; PRLR-L, long form of PRLR; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA; Stat, signal transducer and activator of transcription; WT-Jak2, wild-type Jak2.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

loss of mammary expression of Stat5a or Stat5a/b, substrates of Jak2 in the mammary gland (7), are also characterized by this mammary phenotype (10, 11). Additionally, studies of the immortalized mouse mammary epithelial cell line HC11 further supported the concept that Jak2 mediates PRL-induced Stat5 activation and mammary epithelial differentiation (12).

Importantly, several tyrosine kinases other than Jak2 have been implicated in PRL receptor signaling based on studies in hematopoietic cells and in breast epithelial cells. These tyrosine kinases include the focal adhesion kinase (13), Tec (14), Src (15), and Fyn (16). In addition, cross talk between PRLR and the epidermal growth factor (EGF) receptor (EGFR) has been described in breast cancer cells (17). Likewise, Jak2-dependent cross talk between PRLR and ErbB2 has been described (18). In contrast, evidence has been presented for Jak2-independent activation of Src by PRL (15). Furthermore, PRL signaling to the Erk1/2 kinases has recently been detected in immortalized Jak2-null mouse mammary epithelial cells (68), establishing proof that PRL in some instances can signal independently of Jak2. Insight into the PRLR signaling pathways in normal and malignant mammary epithelial cells will be critical for clarifying the roles of PRL as a promoter of mammary tumor formation on the one hand (reviewed in Ref. 19), and the more recently proposed pro-differentiation role of PRL in established human breast cancer on the other hand (20, 21).

In human breast cancer lines, pro-differentiation effects of the PRL-Jak2-Stat5 signaling pathway have been documented that include promotion of homotypic adhesion and suppression of invasive characteristics (20, 21). Consistent with this notion, activated Stat5 in human breast cancer tissues was associated with a favorable prognosis, and Stat5 was found to be frequently inactivated during metastatic progression (22). Likewise, active Stat5 in human breast cancer tissue correlated positively with histological differentiation (23, 24). On the other hand, several lines of evidence support a role of PRL in rodent mammary tumor formation. PRL-overexpressing transgenic mice have an increased incidence of mammary tumors (25, 26), whereas PRL knock-out mice have a reduced incidence of mammary tumors (27). In breast cancer cells grown under certain culture conditions, PRL has been shown to induce proliferation (28–30) and prolong survival (31). It has been estimated that 70–95% of human breast cancers express PRLR (32, 33), and elevated serum PRL levels have been associated with an increased risk of breast cancer in postmenopausal women (34, 35). Provided the evidence suggesting that the PRL-induced Jak2-Stat5 pathway mediates differentiation and growth inhibition of mammary epithelial cells, and that active Stat5 is progressively lost in breast cancer, it is possible that PRL acts through one or more alternate pathways to promote tumor growth and progression. For instance, in addition to activating the Stat5 pathway, PRL is capable of activating Ras-Raf-Erk1/2, phosphoinositide 3-kinase-Akt, and protein kinase C pathways (reviewed in Ref. 19). It is also possible that PRL signaling differs between normal breast epithelia and early-stage and advanced breast cancer. To identify new molecular targets for chemoprevention and therapy of breast cancer, it will therefore be important to clarify the roles of the various PRL signaling pathways in the development and progression of breast cancer.

We now report that in a subset of human breast cancer cell lines, Jak1 is activated by PRLR signaling. Jak1 was activated with rapid time and dose kinetics that were indistinguishable from Jak2, raising the possibilities of parallel and independent signaling by PRLR by the two Jak kinases, or that Jak1 activation represented a novel branch of PRL signaling pathways downstream of Jak2. Multiple independent strategies to disrupt PRL activation of Jak2, including Jak2 mRNA silencing, dominant-negative Jak2, and pharmacological Jak2 inhibition, consistently disrupted PRL activation of Jak1. Thus, we conclude that the novel PRLR-mediated activation of Jak1 in breast cancer cell lines occurs by a Jak2-dependent mechanism. Importantly, Jak1 was required for downstream activation of ERK and Stat3, for maximal tyrosine phosphorylation of PRLR, and for maximal activation of Jak2, Stat5a, Stat5b, and Akt. The identification of PRLR activation of Jak1 in human breast cancer cells provides a new proximal branching point of PRL signaling pathways that may be important for understanding the biological roles of PRL in development and progression of human breast cancer.

RESULTS

PRL Induces Tyrosine Phosphorylation and Enzymatic Activation of Jak1 in Human Breast Cancer Cell Lines

The ability of PRL to activate each of the four Janus kinases was examined in a panel of breast cancer and near-normal mammary epithelial cell lines, as well as a prostate cancer cell line. After treatment with or without 20 nM PRL for 15 min, cell lysates were collected and Jak proteins were immunoprecipitated and subjected collectively to antiphosphotyrosine immunoblotting. Intriguingly, PRL induced marked tyrosine phosphorylation of Jak1 along with Jak2 in T47D, MCF7, and SKBR3 breast cancer cell lines (Fig. 1A). However, PRL-induced phosphorylation of Jak1 was not observed in the near-normal mammary epithelial cell lines HC11, MCF10A, ME16C, and HBL-100 or in the prostate cancer cell line CWR22Rv1 under these conditions (Fig. 1A). Whereas PRL stimulated Jak2 phosphorylation in HC11 and CWR22Rv1 cells, PRL did not induce phosphorylation of either Jak2 or Jak1 in MCF10A, ME16C, or HBL-100. In addition, PRL induced modest phosphorylation of Tyk2 in T47D cells but not in the other cell lines tested. Because the extent of PRL-induced tyrosine phosphorylation of

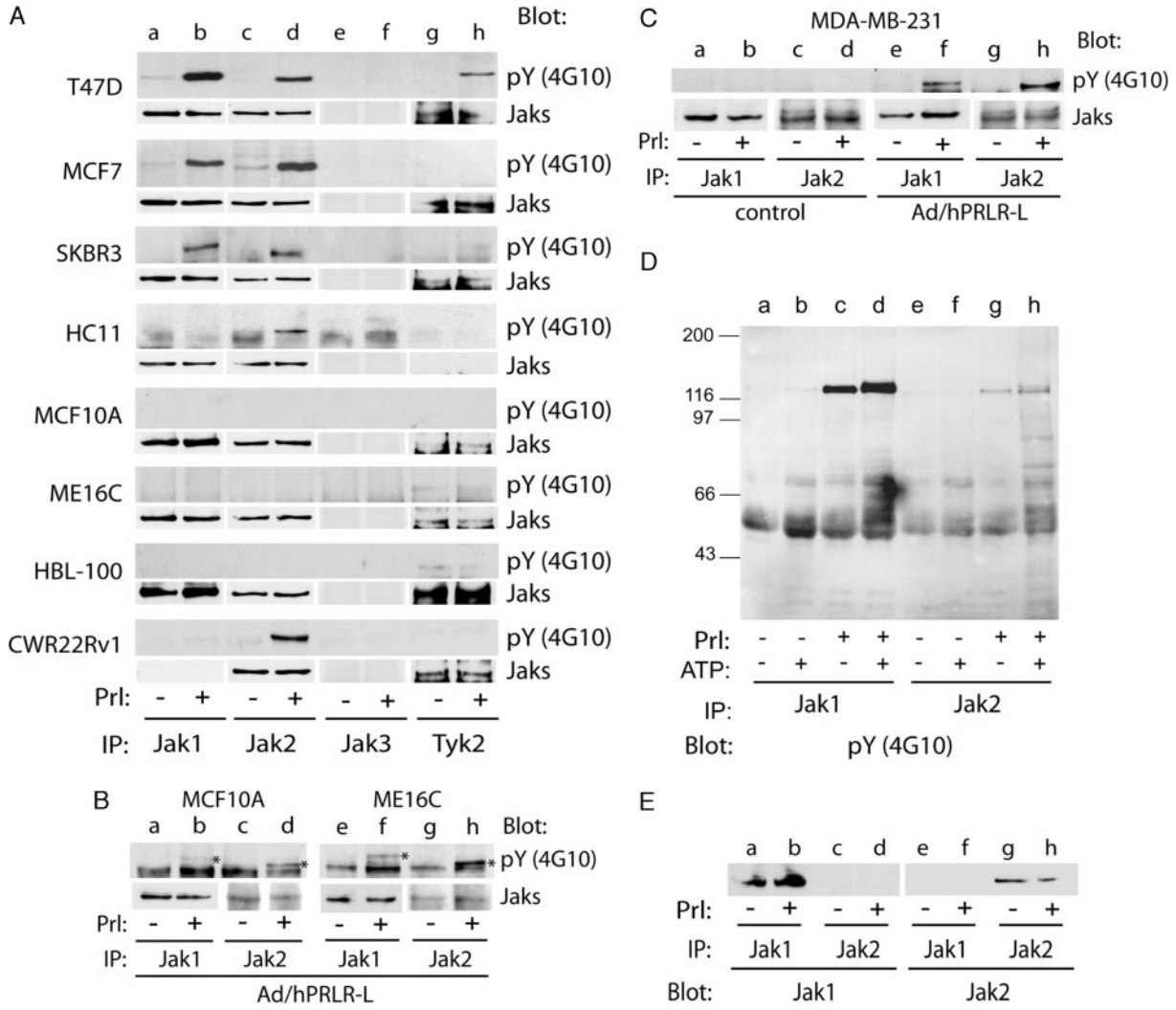

Fig. 1. PRL Induces Tyrosine Phosphorylation and Enzymatic Activation of Jak1 in Human Breast Cancer Cell Lines

A, Antiphosphotyrosine (pY) immunoblots of immunoprecipitated Jak proteins (Jak1, Jak2, Jak3, and Tyk2) from lysates of T47D, MCF7, and SKBR3 human breast cancer cells, HC11, MCF10A, ME16C, and HBL-100 near-normal mammary epithelial cells, and CWR22Rv1 human prostate cancer cells. The cells had been incubated with (+) or without (−) 20 nM hPRL for 15 min at 37 C. Jak protein expression levels in same panel of cell lines were determined. B and C, Anti-pY or total protein immunoblots of immunoprecipitated Jak1 or Jak2 from MCF10A or ME16C cells (B) or MDA-MB-231 cells (C). The cells had been infected without or with hPRLR-L-expressing adenovirus before incubation with (+) or without (−) 20 nM hPRL for 15 min at 37 C. D, PRL-induced activation of Jak1 and Jak2 analyzed by *in vitro* autophosphorylation kinase assay using unlabeled ATP and anti-pY immunoblotting to detect incorporated phosphate on tyrosine residues. Jak1 or Jak2 immunoprecipitated from lysates of T47D cells that had been incubated with (+) or without (−) 10 nM hPRL for 2 min at 37 C were washed and subsequently incubated for 20 min at 37 C in the absence (−) or presence (+) of 15 μM unlabeled ATP. E, Immunoblot confirming specificity of Jak1 and Jak2 antibodies. IP, Immunoprecipitation.

Jak1 was comparable to that of Jak2 in multiple cell lines and markedly stronger than that of Tyk2, which was only induced in T47D cells, subsequent work focused on PRL activation of Jak1. Expression levels of individual immunoprecipitated Jak proteins were assessed in each cell line (Fig. 1A). Notably, Jak1 levels were undetectable in the PRL-responsive CWR22Rv1 cell line, consistent with lack of PRL activation of Jak1 in this cell line.

Several published reports have suggested that near-normal human mammary epithelial cells lines express low levels of the PRLR (36, 37), a potential explanation for the lack of PRL-induced Jak activation. To overcome the problem of low PRLR expression in MCF10A and ME16C cell lines, and to effectively reconstitute PRLR expression and determine whether PRLR would restore PRL phosphorylation of both Jak2 and Jak1, we generated an adenovirus for gene delivery of the long form of the human PRLR (hPRLR-L). Expression of exogenous hPRLR-L by adenoviral gene delivery restored PRL phosphorylation of Jak1 and Jak2 in both MCF10A and ME16C cells (Fig. 1B). In

addition, MDA-MB-231 breast cancer cells express low levels of PRLR (36), and PRL did not induce phosphorylation of Jak1 or Jak2 in these cells (Fig. 1C). Like MCF10A and ME16C, however, expression of exogenous hPRLR-L by adenoviral gene delivery reconstituted PRL-Jak1 and PRL-Jak2 phosphorylation in MDA-MB-231 cells (Fig. 1C). These results indicate that it is the lack of PRLR expression that hinders PRL responsiveness in these cell lines.

To determine whether phosphorylation of Jak1 by PRL was associated with increased enzymatic activity of the Jak1 kinase in breast cancer cells and did not simply reflect passive collateral phosphorylation, *in vitro* autophosphorylation Jak kinase assays were performed as previously described (2, 38). T47D cells were incubated briefly with or without PRL for 2 min before Jak1 and Jak2 were individually immunoprecipitated from cell lysates and incubated for 20 min in the presence or absence of 15 μM unlabeled ATP at 37 C, and separated on SDS-PAGE. Antiphosphotyrosine immunoblotting demonstrated that only after PRL stimulation did Jak1 and Jak2 both further incorporate phosphate on tyrosine residues during incubation with ATP, verifying that both kinases became enzymatically activated by PRL (Fig. 1D). Whereas PRL activated both Jak1 and Jak2 in T47D cells, antibodies to Jak1 did not bring down Jak2 and vice versa (Fig. 1E), confirming the specificity of the Jak1 and Jak2 antibodies used for immunoprecipitation in these studies and allowing us to study the phosphorylation and activation of the two kinases separately.

PRL Induces Tyrosine Phosphorylation of Jak1 and Jak2 with Similar Time Kinetics and Dose Responses

To determine the rate and dose at which Jak1 becomes maximally phosphorylated by PRL in T47D cells, time course and dose-response experiments were conducted. In cells treated with 100 nM PRL for up to 20 min, Jak1 and Jak2 phosphorylation was examined in parallel by immunoprecipitation combined with immunoblot analysis. Both Jak1 and Jak2 were markedly phosphorylated already within 1.3 min of PRL treatment, and both proteins reached maximum phosphorylation between 2.5 and 5 min (Fig. 2A). When the PRL concentration was varied up to 100 nM, both Jak1 and Jak2 became maximally phosphorylated at 10 nM of PRL (Fig. 2B). Densitometric analysis of immunoblots representing three independent dose-response and time course experiments demonstrated indistinguishable time and dose curves for PRL activation of Jak1 and Jak2 ($EC_{50} \sim 4$ nM, Fig. 2C). The similar kinetics of activation of Jak1 and Jak2 indicated that the two kinases were equally sensitive to PRL and that activation of Jak1 occurred without delay and simultaneously with activation of Jak2.

Overexpression of Jak2 Does Not Interfere with PRL Activation of Jak1

Because Jak1 and Jak2 were activated concurrently, we wanted to determine whether PRL activation of Jak1 was due to relatively low levels of Jak2 in breast cancer cells. Increasing levels of wild-type Jak2 (WT-Jak2) were introduced into T47D cells by adenoviral delivery [multiplicity of infection (MOI) 10, 20, and 40] to saturate the intracellular Jak2-binding interface of the PRLR to determine whether PRL activation of Jak1 would increase or diminish. When no virus or LacZ control virus was administered to the cells (Fig. 3A, lanes a–d), PRL induced tyrosine phosphorylation of both Jak1 and Jak2 (panels 1 and 3). Overexpression of WT-Jak2 did not block PRL-induced Jak1 phosphorylation (Fig. 3A, panel 1, lanes e–j), suggesting that a relative Jak2 deficiency was not the cause of PRL activation of Jak1. At the highest MOI (Fig. 3A, lanes i–j), both Jak1 and Jak2 were constitutively activated, probably due to excessive hyperactivation of overexpressed Jak2. Note also that adenoviral epitope-tagged WT-Jak2 migrates slower than Jak1 and is present in Jak1 immunoprecipitates as a contaminant due to the highly elevated levels of expression after adenoviral gene delivery (Fig. 3A, panel 1, lanes e–j).

To explore the dependency of PRL activation of Jak1 on Jak2, we first used Jak2 conditional knockout mice (7, 39) to derive chemically induced mammary tumor cell lines that lack Jak2 and their isogenic wild-type controls. For this purpose, we generated mice that carry the Jak2 conditional knockout (floxed) allele in a greater than 93% FvB background. Animals that carry two Jak2 floxed alleles were treated three times with 7,12-dimethylbenz(A) anthracene (DMBA) via gastric gavage, and mammary tumors originated approximately 6–12 months after the chemical treatment. Primary tumor cell lines were derived from individual DMBA-induced mammary tumors of conditional Jak2 knockout mice (JDM cells) as described in *Materials and Methods*. Primary cells of one tumor that exhibited perfect epithelial morphology (JDM1 cells) were infected with the pBabe-puro retrovirus control vector (JDM1.1) or the pBabe-Cre-puro retroviral construct (JDM1.2), which allows a virtually complete deletion of floxed loci after puromycin selection (40). $Jak2^{fl/fl}$ JDM1.1 cells expressed both endogenous Jak1 and Jak2, whereas Jak2-deficient ($Jak2^{-/-}$) JDM1.2 cells expressed Jak1 but lacked the Jak2 protein (Fig. 3B, panels 2 and 4, *left*). Regardless of Jak2 expression status, PRL failed to activate either Jak2 or Jak1 in JDM1.1 and JDM1.2 cells (Fig. 3B, panels 1 and 3, *left*). To overcome the problem of low PRLR expression in the JDM cell lines, and to effectively reconstitute PRLR expression and determine whether PRLR would restore PRL activation of both Jak2 and Jak1, we infected the cells with adenovirus expressing hPRLR-L. Interestingly, expression of exogenous hPRLR-L by adenoviral gene delivery restored PRL

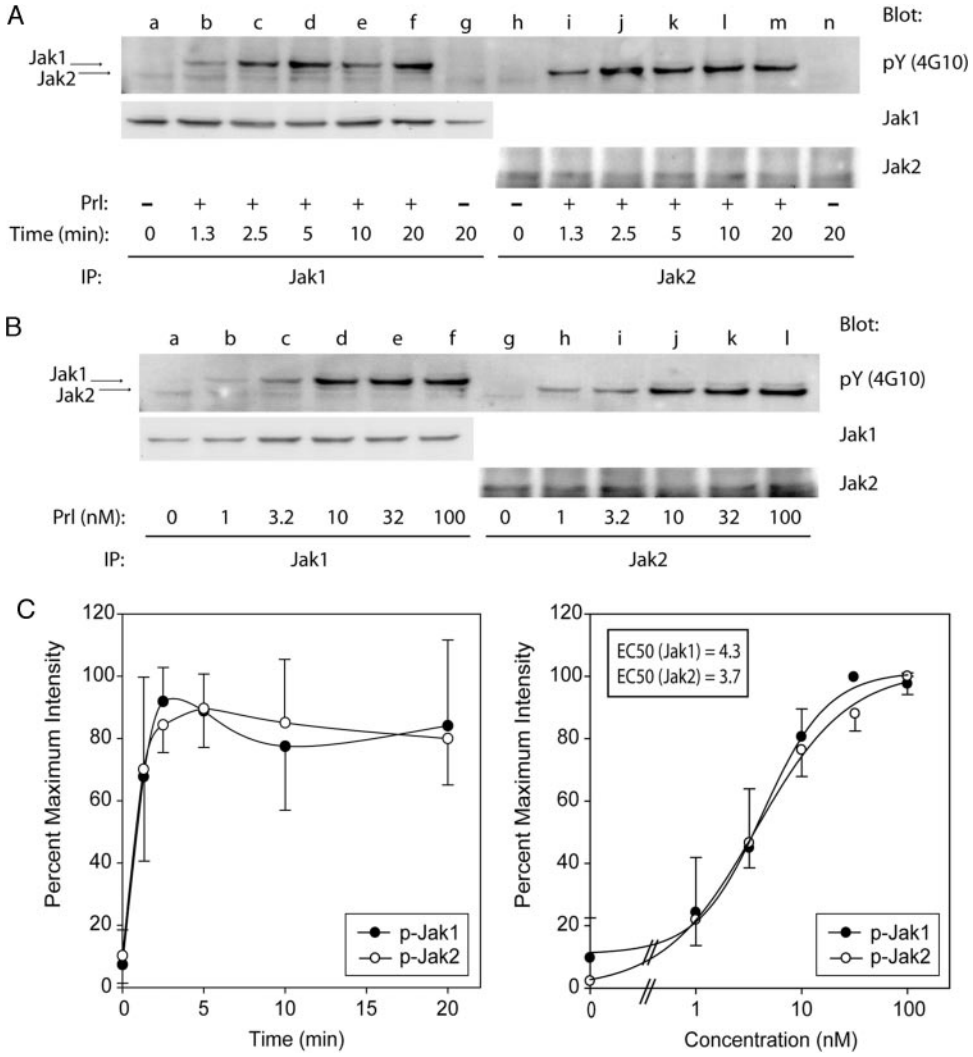

Fig. 2. PRL Induces Tyrosine Phosphorylation of Jak1 and Jak2 with Similar Time Kinetics and Dose Responses in T47D Cells

A and B, Anti-pY or total protein immunoblots of immunoprecipitated Jak1 and Jak2 from lysates of T47D cells. A, The cells had been incubated with 100 nM hPRL for varying times up to 20 min at 37 C, or B) with varying concentrations of hPRL up to 100 nM for 15 min. C, Densitometric analysis corresponding to time-course and dose-response immunoblots. *Graphs* represent the average of three independent experiments. IP, Immunoprecipitation; pY, phosphotyrosine.

activation of Jak2 but not Jak1 in JDM1.1 cells (Fig. 3B, panels 1 and 3, *right*). No activation of Jak1 was seen in Jak2-deficient JDM1.2 cells. These initial observations suggested that PRLR and Jak2 are required but not sufficient for PRL activation of Jak1.

PRL-Induced Jak1 Activation in T47D Cells Is Dependent on Jak2

To examine whether PRL-induced Jak1 activation in human breast cancer cells was independent of or required Jak2 activation, we employed several methods to suppress Jak2 activation in T47D cells. First, five distinct candidate Jak2 short hairpin RNAs (shRNAs) cloned into the pLKO.1-puro Lentiviral expression plasmid (Sigma Chemical Co., St. Louis, MO) were tested for efficacy to knock down endogenous Jak2 mRNA levels in COS-7 cells. Three of five candidate shRNA sequences, when transiently transfected into COS-7 cells, reduced Jak2 mRNA expression levels by 50% or more of control levels, as determined by quantitative real-time PCR (shRNA-79, shRNA-80, and shRNA-81; Fig. 4A). Lentiviral particles expressing Jak2 shRNA-80 were selected for further study and resulted in a dose-dependent reduction of Jak2 mRNA levels in T47D cells, achieving a 65% knockdown at the highest dose of virus (MOI = 125) as determined by quantitative RT-PCR (qRT-PCR) (Fig. 4B). In contrast, Jak1 mRNA levels were not reduced in cells treated with Jak2 shRNA-80, demonstrating target specificity of this shRNA. Furthermore, Jak2 mRNA knockdown by shRNA-80 lentiviral infection at MOI 125 resulted in highly effective reduction of Jak2 protein expression (Fig. 4C, panel 4, lanes e–f). As ex-

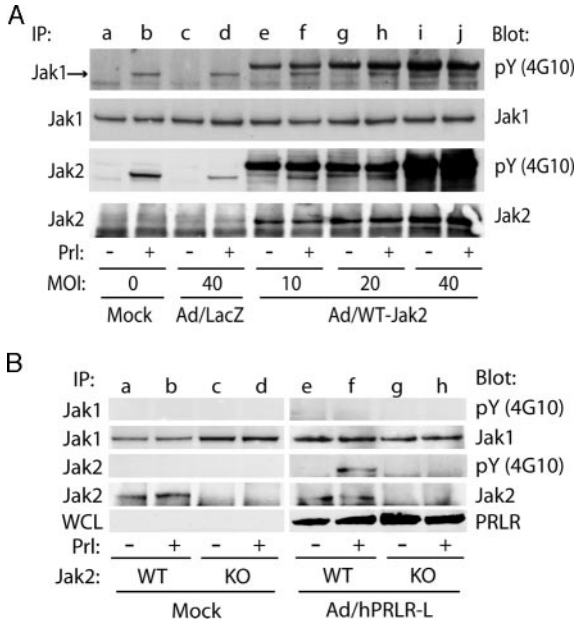

Fig. 3. Overexpression of Jak2 Does Not Interfere with PRL Activation of Jak1

A, Cells were infected with no virus, control LacZ adenovirus, or increasing MOI (10, 20, and 40) of WT-Jak2 adenovirus before stimulation with (+) or without (−) 20 nM hPRL for 15 min. Cellular lysates were immunoprecipitated for Jak1 or Jak2, and immunoblotting was performed using antibodies against phosphotyrosine (pY), Jak1, or Jak2. Note: Due to its high expression levels, some adenoviral, epitope-tagged Jak2 was pulled down nonspecifically with Jak1 immunoprecipitation and is visible as a slower-migrating band in the pY blot (*top panel*, lanes e–j, *upper band*). B, Anti-pY and total Jak protein blots of immunoprecipitated Jak1 and Jak2 proteins from lysates of two matched, isogenic DMBA-induced mouse mammary tumor cell lines JDM1.1 and JDM1.2 (Jak2 WT and Jak2 KO) infected without (*left panels*) or with (*right panels*) hPRLR-L-expressing adenovirus. Cells had been incubated with (+) or without (−) 20 nM hPRL for 15 min at 37 C before lysate harvesting. IP, Immunoprecipitation; KO, knockout.

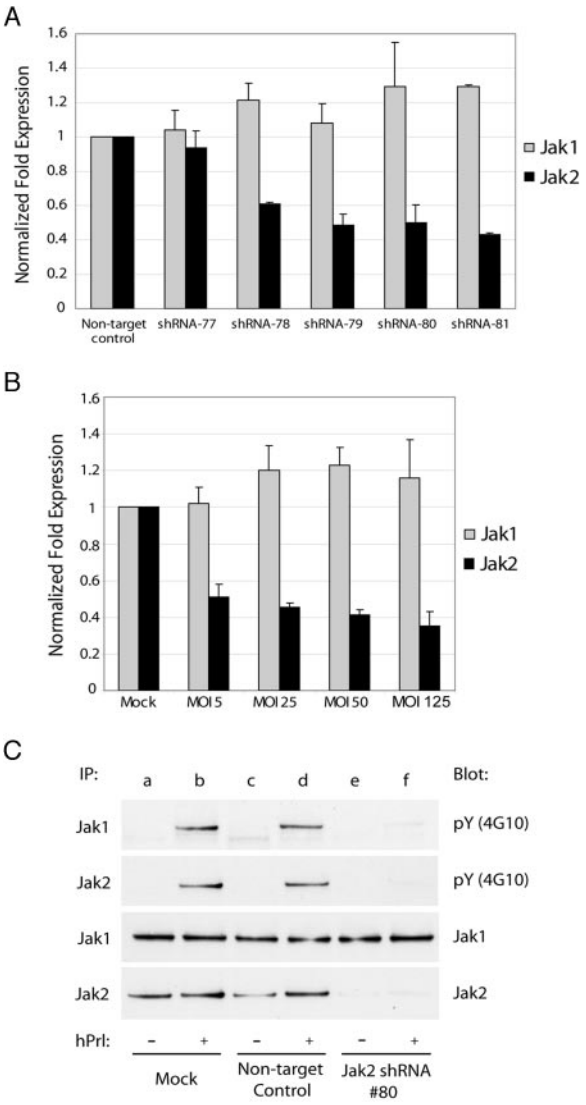

Fig. 4. PRL Activation of Jak1 in T47D Cells Is Blocked by shRNA Suppression of Jak2

A, COS-7 cells were transiently transfected with nontarget control shRNA plasmid or one of five Jak2-targeted shRNA plasmids. RNA was isolated 48 h after transfection, and quantitative real-time PCR was performed using primers to detect GAPDH (internal control), Jak1, and Jak2 for each sample. B, T47D cells were infected with lentiviral particles expressing Jak2 shRNA-80 at increasing MOIs. RNA was isolated 72 h after infection, and qRT-PCR was performed as in panel A. C, T47D cells were treated with no virus, nontarget control lentivirus, or Jak2 shRNA-80-expressing lentivirus before serum starvation and treatment with (+) or without (−) 20 nM hPRL for 15 min. Cellular lysates were immunoprecipitated for Jak1 or Jak2, and immunoblotting was performed using antibodies against phosphotyrosine (pY), Jak1, or Jak2. IP, Immunoprecipitation.

pected, no phosphorylated Jak2 was detectable after PRL treatment under these conditions (Fig. 4C, panel 2, lanes e–f). Although Jak1 protein levels were not reduced by Jak2 shRNA (Fig. 4C, panel 3, lanes e–f), PRL was unable to induce Jak1 phosphorylation in the absence of Jak2 activation (Fig. 4C, panel 1, lanes e–f), providing a first line of evidence that Jak2 is required for PRL activation of Jak1.

As a second strategy to determine whether Jak2 is required for PRL activation of Jak1, pretreatment of T47D cells with 50 μM of the Jak2 inhibitor AG490 inhibited PRL activation of Jak2 and also inhibited PRL activation of Jak1 (Fig. 5A, panels 1 and 3, lanes g–h). In contrast, oncostatin-M (OSM) remained capable of activating Jak1 at 50 μM AG490 (Fig. 5A, panel 1, lane i), indicating that AG490 specifically inhibited Jak2 and not Jak1 at this concentration. Nonspecific inhibition of Jak1 occurred when cells were pretreated with 75 μM AG490, because Jak1 activation by OSM was also blocked at this higher concentration of inhibitor (Fig. 5A, panel 1, lanes j–l). These pharmacological experiments provided further support for a Jak2-dependent PRL activation of Jak1.

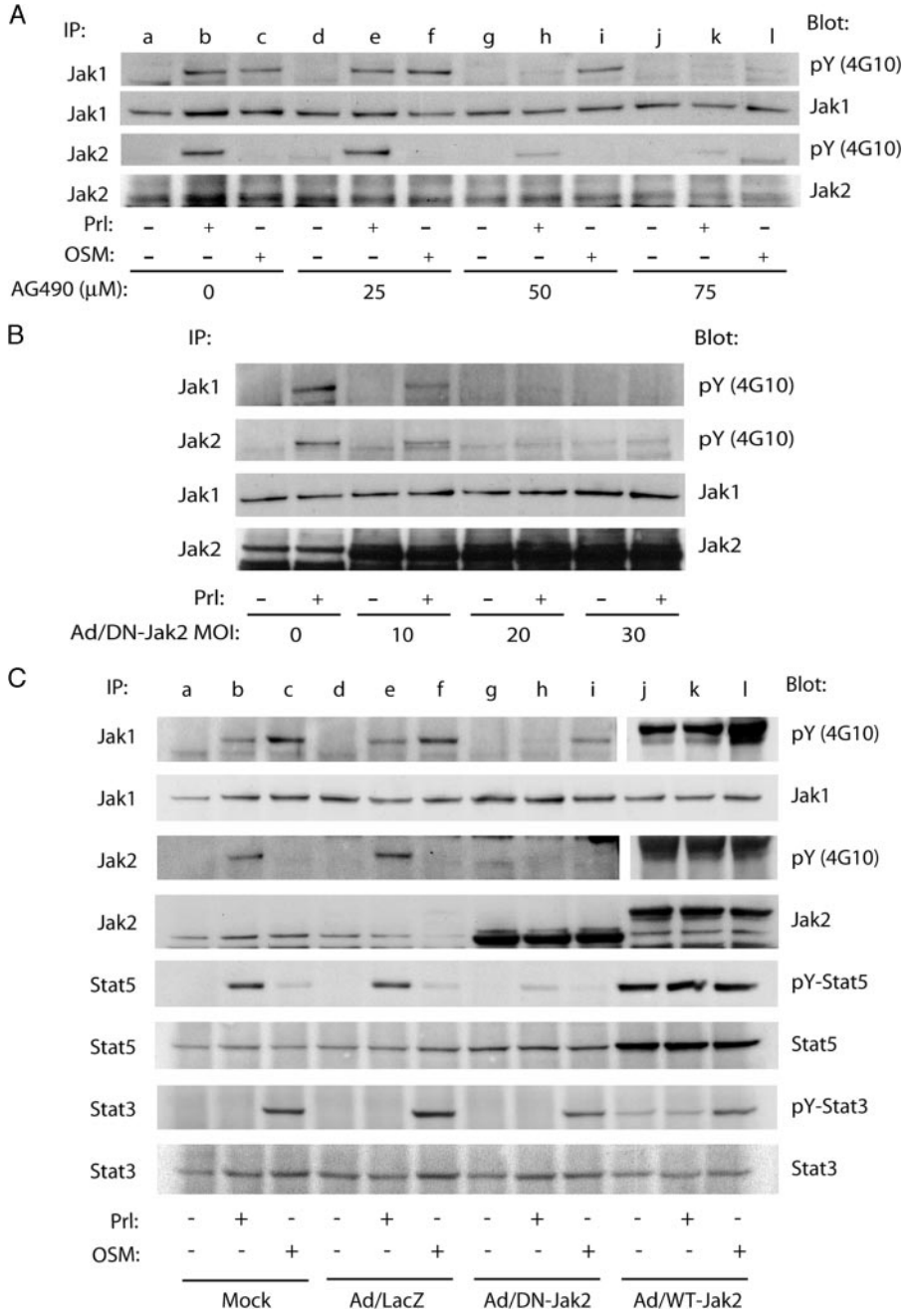

Fig. 5. PRL Activation of Jak1 in T47D Cells Is Blocked by Inhibition of Jak2

A, Cells were treated with increasing concentrations of the Jak2 kinase inhibitor AG490 in serum-free media for 16 h before stimulation with or without 20 nM hPRL or 20 nM hOSM, or B) cells were infected with no virus or increasing MOI of DN-Jak2 adenovirus for 24 h before serum starvation and stimulation with or without 20 nM hPRL, or C) cells were infected with no virus, control LacZ adenovirus, DN-Jak2 adenovirus, or WT-Jak2 adenovirus for 24 h before serum starvation and stimulation with or without 20 nM hPRL or 20 nM hOSM. Cellular lysates were immunoprecipitated for Jak1, Jak2, Stat5, or Stat3, and immunoblotting was performed using antibodies against phosphotyrosine (pY), Jak1, Jak2, tyrosine-phosphorylated Stat5 (pY-Stat5), total Stat5, tyrosine phosphorylated Stat3 (pY-Stat3), or total Stat3 as indicated. IP, Immunoprecipitation.

A third independent method to determine whether Jak2 activation is required for PRL activation of Jak1 in breast cancer cells involved overexpression of a kinase-deficient dominant-negative Jak2 mutant (DN-Jak2) by adenoviral delivery into T47D cells. Levels of PRL activation of Jak2 and Jak1 were inhibited in a dose-dependent manner in cells treated with DN-Jak2 adenovirus, with partial suppression at MOI 10 and complete suppression at MOI 20 and 30 (Fig. 5B, panels 1 and 2). The DN-Jak2 did not affect levels of

Jak1 (Fig. 5B, panel 3), demonstrating that loss of Jak1 signal was not caused by loss of Jak1 protein. To determine whether the decrease in PRL activated Jak1 was due specifically to the decrease in activated Jak2 and not cytopathic effect of virus, T47D cells were infected with no virus (mock control), LacZ control adenovirus (MOI = 20), DN-Jak2 adenovirus (MOI = 20), or WT-Jak2 adenovirus (MOI = 20) before stimulation with or without 20 nM PRL, or with 20 nM OSM as a separate control for pathway specificity. In both mock and LacZ control cells, Jak1 became activated upon treatment with PRL or OSM, and Jak2 became activated by PRL but not by OSM (Fig. 5C, panels 1 and 3, lanes a–f). When DN-Jak2 was overexpressed, PRL activation of both Jak2 and Jak1 was blocked, whereas OSM activation of Jak1 remained detectable (Fig. 5C, panels 1 and 3, lanes g–i). Downstream Stat activation status reinforced PRL and OSM pathway specificity in this experiment. Stat5 became phosphorylated with PRL treatment in mock and LacZ control cells, but not in DN-Jak2-overexpressing cells in which Jak2 activation was blocked (Fig. 5C, panel 5, lanes a–i). Overexpression of WT-Jak2 led to constitutive activation of Stat5 (Fig. 5C, panel 5, lanes j–l), which is consistent with its role as the Stat5 tyrosine kinase in mammary epithelial cells (7, 12). Furthermore, OSM activation of Jak1 and Stat3 remained intact in both DN-Jak2- and WT-Jak2-overexpressing cells (Fig. 5C, panels 1 and 7), indicating that the OSM-Jak1-Stat3 pathway was not inhibited when activated Jak2 levels were blocked. However, a modest reduction in OSM-induced Jak1 and Stat3 signals may be due to a minor role of Jak2 in OSM signaling (41). Collectively, these data suggest that Jak2 activation is required for PRL activation of Jak1. In fact, three independent molecular strategies to selectively suppress Jak2 activity, including gene knockdown, dominant-negative mutant, and small molecular inhibitor, consistently showed that Jak2 activation was required for PRL activation of Jak1.

Jak1 Is a Central Mediator of PRL Signaling in T47D and SKBR3 Breast Cancer Cells

To determine the role of Jak1 as a mediator of PRL signaling in breast cancer cells, four distinct candidate Jak1 shRNAs cloned into the pLKO.1-puro Lentiviral expression plasmid (Sigma) were tested for efficacy to knock down endogenous Jak1 mRNA levels in COS-7 cells. Two of four shRNA constructs, when transiently transfected into COS-7 cells, reduced Jak1 mRNA expression levels by 50% or more of control levels, as determined by quantitative real-time PCR (shRNA-2 and shRNA-5, Fig. 6A). Lentiviral particles expressing Jak1 shRNA-2 and shRNA-5 were selected for further study and resulted in a dose-dependent reduction of Jak1 mRNA levels in T47D cells, achieving a 93% knockdown with shRNA-2 (Fig. 6B, MOI = 50) and an 85% knockdown with shRNA-5 (Fig. 6C, MOI = 50) as determined by qRT-PCR. Knockdown of Jak1 mRNA by shRNA-2 or shRNA-5 lentiviral infection resulted in a highly effective reduction of Jak1 protein expression in T47D and SKBR3 cells (Fig. 6D and 6E, respectively). In each of these breast cancer cell lines, blocking PRL activation of Jak1 by shRNA lentiviral infection blocked PRL activation of ERK and Stat3 and partially reduced PRL activation of Jak2, Akt, Stat5a, and Stat5b as well as tyrosine phosphorylation of PRLR (Fig. 6, D and E). Importantly, PRL activation of Jak2 was least sensitive to Jak1 knockdown, consistent with the role of Jak2 as a primary PRLR-activated tyrosine kinase. Semiquantitative densitometry is provided to compare the effects of Jak1 knockdown on PRL signals in each cell line (Fig. 6F). Jak1 loss did not affect protein levels of PRLR, Jak2, or other signaling components, suggesting that the effect of Jak1 knockdown is direct. However, it cannot be excluded that some of the effect is indirect, *e.g*. up-regulation of a Jak2 phosphatase, after Jak1 loss. The data suggest a role for Jak1 as a broadly acting positive modulator of PRLR-Jak2 signals, with some signals being more dependent than others.

DISCUSSION

This report describes novel and marked activation of the Jak1 tyrosine kinase by PRL, in parallel with classical activation of Jak2, in several human breast cancer cell lines, including ER-positive T47D and MCF7 and ER-negative SKBR3. PRL activated Jak1 with the same rapid and sensitive time and dose kinetics as Jak2, suggesting that PRL activation of Jak1 is a signaling event proximal to the PRLR. Importantly, PRL activation of Jak1 occurred through a Jak2-dependent mechanism, as evidenced by multiple independent experimental strategies to block PRL activation of Jak2, including use of Jak2 gene knockdown, dominant-negative Jak2, and pharmacological Jak2 inhibition. Finally, selective Jak1 gene knockdown effectively disrupted PRL activation of ERK and Stat3 and partially suppressed PRL activation of Jak2, Akt, Stat5a, and Stat5b without affecting expression levels of these proteins. Collectively, the data from several breast cancer cell lines are consistent with the novel concept that recruitment of Jak1 by PRL may serve to both amplify existing Jak2-dependent signals and to establish additional, branching signaling pathways.

Jak1 as a Transducer of PRL Effects

The present data indicate a major role of Jak1 as an enhancer of PRL signals in a subset of human breast cancer cell lines. In addition, the dependence of PRL-activated ERK and Stat3 pathways on Jak1 raises the possibility that Jak1 represents a new and conditional branching point of the PRLR signaling network that is active in breast cancer. The finding of marked and novel PRL activation of Jak1 in mul-

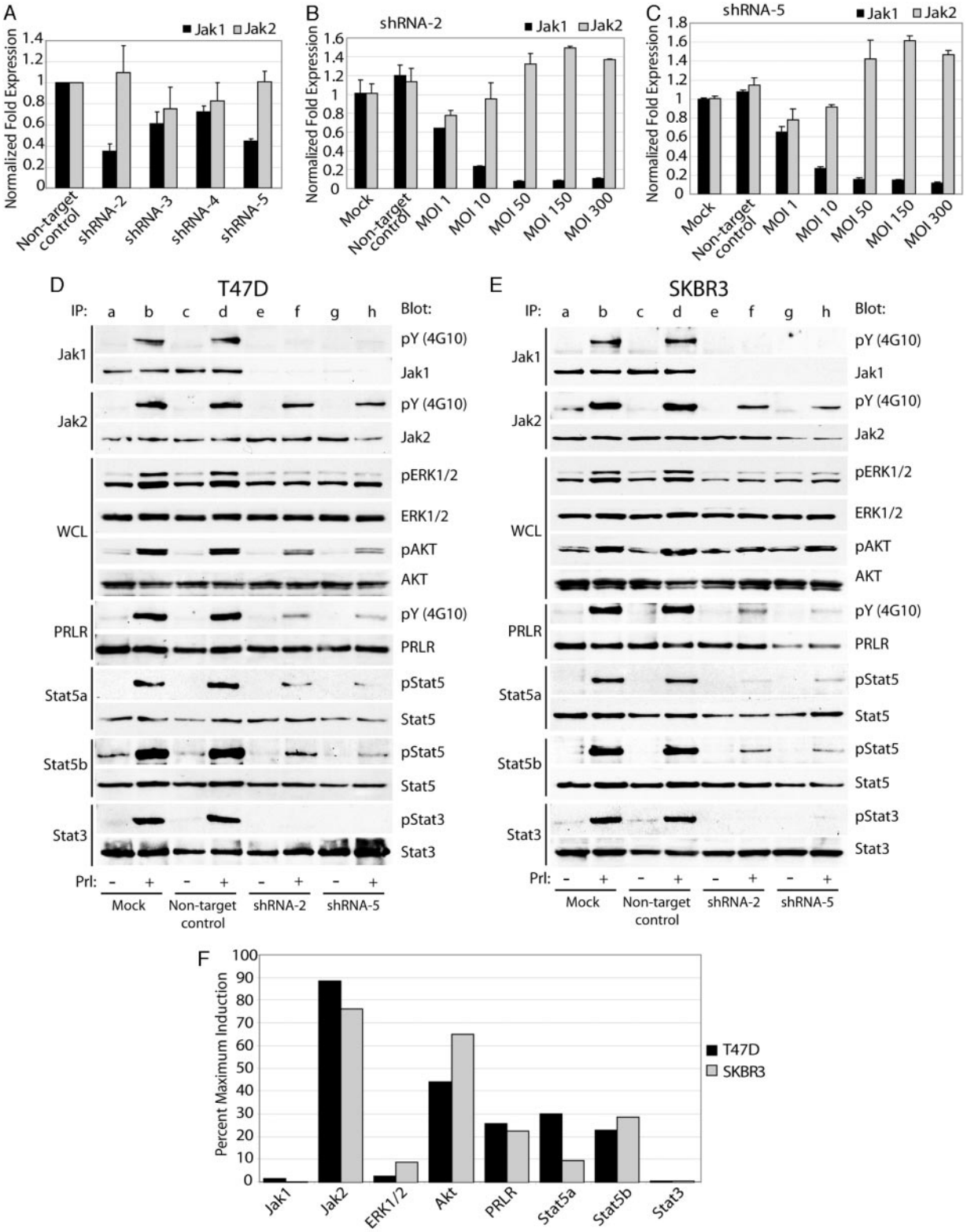

Fig. 6. Jak1 Is a Central Mediator of PRL Signaling in T47D and SKBR3 Breast Cancer Cells

A, COS-7 cells were transiently transfected with nontarget control shRNA plasmid or one of four Jak1-targeted shRNA plasmids. RNA was isolated 48 h after transfection, and quantitative real-time PCR was performed using primers to detect GAPDH (internal control), Jak1, and Jak2 for each sample. B and C, T47D cells were infected with lentiviral particles expressing Jak1 shRNA-2 (B) or Jak1 shRNA-5 (C) at increasing MOIs. RNA was isolated 72 h after infection, and qRT-PCR was performed as in panel A. D and E, T47D cells (D) or SKBR3 cells (E) were treated with no virus, nontarget control lentivirus, or Jak1 shRNA-2- or

tiple human breast cancer cell lines and data consistent with novel roles of Jak1 as a transducer of PRL signals raise several issues relevant to understanding PRL-induced signal transduction and effects in malignant breast epithelia.

Of immediate interest is to establish the biological role of Jak1 as a mediator of PRL effects in breast cancer. Determining the function of Jak1 downstream of PRLR activation in human breast cancer may shed new light on the multiple and controversial roles of PRL in breast cancer, which include regulation of cell proliferation (29, 42–44), survival (31), migration (45), and differentiation, adhesion, and invasion (20, 21). It is possible that Jak1 acts as a conditional modulator of PRL signals and effects, especially affecting the balance of tumor-promoting and prodifferentiation effects of PRL. It will therefore be important to explore the role of Jak1 as a broad enhancer of PRLR signals in human breast cancer, *e.g*. Stat5a, Stat5b, and phosphoinositide 3-kinase-Akt, and the role of Jak1 as a branching node for recruitment of additional signals that may not be activated by Jak2 alone, *e.g*. ERK, Stat3, and possibly other signals such as Src and Tec (46–50). Thus, Jak1 may contribute to PRL signaling and biology by both critically mediating activation of a subset of PRLR pathways, and serve as a general enhancer of other Jak2-mediated PRLR pathways.

From a pharmacological perspective, the novel involvement of Jak1 in PRLR signaling, at least in a subset of breast cancer, may represent a new pharmacological target. Specifically, combined inhibition of Jak2 and Jak1 may synergize to suppress growth and survival-promoting PRL effects in some tumors and be advantageous over inhibition of Jak2 alone. Furthermore, if Jak1-specific pathways were to preferentially mediate tumor-promoting effects of PRL, inhibitors of Jak1 may be useful in breast cancer treatment to preferentially disrupt select PRL-induced signals while having less effect on other signaling pathways. Such a strategy might become clinically relevant if Jak1-dependent modulation of PRL-activated ERK and Stat3 stimulates tumor growth or invasion (20, 46, 51, 52), in contrast to a proposed differentiation-promoting role of the Jak2-Stat5 pathway (12, 20, 21). Obviously, further work to clarify the significance of Jak1 as a mediator of PRL effects on breast cancer cells and proof of principle for involvement in growth regulation will be required before Jak1 can be considered more than a candidate drug target.

The homodimerizing PRLR has long been considered a selective activator of Jak2, similar to the related homodimerizing receptors for GH, erythropoietin, and thrombopoietin (19, 53, 54). Interestingly, an early report suggested that PRL induced minor tyrosine phosphorylation of Jak1 in addition to predominant tyrosine phosphorylation of Jak2 in the PRLR-transfected pro-B cell line Ba/F3 (55). However, we and other groups did not observe such Jak1 phosphorylation by PRL in various hematopoietic cell lines and specifically reported PRL activation of Jak2 but not Jak1 (2, 4, 5, 56, 57). Furthermore, after our detection of marked Jak1 activation in breast cancer cells in the present work, we specifically reinvestigated PRL activation of Jaks in PRLR-expressing Ba/F3 cells, as well as other hematopoietic cell lines (Nb2 and 32D-PRLR cells), and detected exclusive PRL phosphorylation of Jak2 and not of Jak1 (data not shown). Because Jak1 migrates slightly but detectably slower than Jak2 in SDS-PAGE (*e.g*. see Fig. 1A), the lack of separation between Jak2 and the tyrosine-phosphorylated band in the Jak1-immunoprecipitated lanes in the former report (55) suggested that the faint tyrosine-phosphorylated band in the Jak1 immunoprecipitates most likely represented nonspecific capture of minor amounts of Jak2. Importantly, the present observation of marked PRL activation of Jak1 in breast cancer cell lines does not challenge the established role of Jak2 as the primary PRLR-coupled Janus kinase, because Jak1 activation was dependent on PRL activation of Jak2, whereas PRL was capable of activating Jak2 also in the absence of Jak1 (albeit to a somewhat lesser extent).

Mechanism of Activation of Jak1 by PRL

Based on the data presented here, we propose a working model of PRL activation of Jak1 that involves Jak2-dependent activation of Jak1 by direct transphosphorylation, with some degree of reciprocal transphosphorylation of Jak2 by Jak1 after PRLR activation. Several lines of evidence support this model. First, whereas Jak1 activation by PRL was completely dependent on Jak2 activation, Jak2 activation by PRL was only modestly reduced by selective knockdown of Jak1. Second, the indistinguishable time and dose kinetics of activation of Jak2 and Jak1 by PRL are consistent with parallel and direct activation of Jak1 by Jak2. Furthermore, the inability of Jak2 overexpression to suppress PRL-induced Jak1 activation suggested that Jak1 does not compete with Jak2 for direct binding to the

Jak1 shRNA-5-expressing lentivirus before serum starvation and treatment with (+) or without (−) 100 nM hPRL for 10 min. Cellular lysates were immunoprecipitated for Jak1, Jak2, PRLR, Stat5a, Stat5b, or Stat3, and immunoblotting was performed using antibodies against phosphotyrosine (pY), Jak1, Jak2, phospho-ERK1/2, ERK1/2, phospho-Akt, Akt, PRLR, phospho-Stat5a, Stat5, phospho-Stat3, or Stat3 as indicated. WCL, Whole-cell lysate. F, Semiquantitative densitometry illustrating PRL signal inhibition with Jak1 knockdown in T47D and SKBR3 cell lines. *Bars* represent PRL-induced signal expressed as a percent of the maximum signal. IP, Immunoprecipitation.

Box1/2 region of the PRLR (3, 4). More likely, Jak1 is preassociated with a different transmembrane receptor and that PRL-activated Jak2 transactivates Jak1 by collateral cross talk with this second receptor complex. The existence or requirement of such a Jak1-binding receptor in the vicinity of PRLR is supported by the absence of Jak1 activation in HC11 and PRLR-expressing JDM1.1 cells. Despite activation of Jak2 by PRL and abundant expression of Jak1, PRL activated Jak2, but not Jak1, in these two cell lines (Figs. 1A and 3B). Thus, PRLR and Jak2 are required, but are not sufficient, for PRL activation of Jak1. Our working model therefore considers Jak1 activation by PRL to be a conditional signaling event, which may be turned on or off during normal development or during malignant transformation or malignant progression of PRL target cells depending on expression levels or function of a yet-to-be identified Jak1-binding mediator protein.

The nature of such a mediator protein within the new PRLR-Jak2-Jak1 pathway includes several candidates. Jak1-interacting transmembrane receptors such as EGFR/ErbB2 (58, 59), interferon receptors (60, 61), or members of the IL-6 receptor family (*e.g*. gp130, leukemia inhibitory factor receptor, or OSM receptor) (62) may be aggregated in response to PRLR stimulation as a form of collateral signaling or cross talk. Cooperation between PRLR and EGFR (17) or ErbB2 (18) has been reported, and EGFR has been demonstrated to activate Jak1 in A431 human vulval carcinoma and B82L-EGFR mouse sarcoma cells (58, 59). A mechanistic model involving PRLR-Jak2 recruitment of a Jak1-associated transmembrane receptor would be reminiscent of Jak1 and Jak2 cross-phosphorylation that occurs in heterodimerizing receptor complexes such as those activated by interferon-γ (63). The observed rapid coactivation of Jak1 and Jak2 by PRL is consistent with direct cross-phosphorylation of Jak1 by Jak2. Likewise, the lack of PRL activation of Jak1 when Jak2 activity is suppressed indicates that PRL-induced aggregation of PRLR alone is not sufficient to trigger aggregation and activation of Jak1 bound to another receptor. Alternatively, an intracellular kinase such as Src may rapidly phosphorylate and activate Jak1 downstream of PRL activation of Jak2 (50, 64), or other intracellular adaptor molecules may bridge Jak2 and Jak1 in a cell-dependent manner. These molecular mechanisms are currently being explored.

In summary, we report a new PRLR-Jak2-Jak1 signaling axis based on consistent observations in several human breast cancer cell lines. Jak1 may serve a dual role as a general enhancer of PRL-induced signals, as well as a recruiter of additional Jak1-dependent signals. Ongoing work aims to determine the role of Jak1 in PRL biology and signaling in breast cancer and to further investigate the molecular mechanisms underlying PRL-Jak2 activation of Jak1.

MATERIALS AND METHODS

Materials and Antibodies

Recombinant human PRL (AFP795) was provided by Dr. A. F. Parlow under the sponsorship of the National Hormone and Pituitary Program. Monoclonal antiphosphotyrosine antibody 4G10 and polyclonal rabbit antiserum Jak2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antiphosphotyrosine-Stat5 antibody (AX1) and polyclonal rabbit antisera to Jak1, Jak3, Tyk2, Stat5a, Stat5b, and Stat3 were provided by Advantex BioReagents (Conroe, TX). Polyclonal rabbit antiserum to phosphotyrosine-Stat3, and monoclonal mouse antibodies to phosphoserine-Akt, phosphothreonine/tyrosine-ERK1/2, phosphotyrosine-Stat3, and Akt were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal mouse Jak1, Tyk2, ERK, and Stat5 antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Monoclonal mouse Jak2 antibody was purchased from Biosource (Camarillo, CA). Monoclonal mouse PRLR antibody was purchased from Zymed Laboratories, Inc. (San Francisco, CA). Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The Jak2 inhibitor AG490 was purchased from Calbiochem (San Diego, CA). Human OSM was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Cell Lines

Human breast cancer cell lines T47D and SKBR3 [American Type Culture Collection (ATCC), Manassas, VA] and prostate cancer cell line CWR22Rv1 (kindly provided by Dr. Thomas Pretlow and colleagues, Case Western Reserve University, Cleveland, OH) were grown in RPMI-1640 medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Biofluids), and penicillin-streptomycin (50 IU/ml and 50 μg/ml respectively; Biofluids). The mouse mammary epithelial cell line HC11 (65) was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, insulin (5 μg/ml; Sigma), EGF (10 ng/ml), and penicillin-streptomycin (50 UI/ml and 50 μg/ml, respectively), as previously described (12). The human telomerase reverse transcriptase-immortalized human mammary epithelial cell line ME16C (ATCC) was grown in mammary epithelial growth medium (Clonetics Corp., San Diego, CA) with penicillin-streptomycin (50 UI/ml and 50 μg/ml, respectively) but without gentamycin-amphotericin B. MCF-7, HBL-100, MDA-MB-231, and COS-7 cell lines were grown in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively). The MCF10A cell line (ATCC) was grown in DMEM:F12 media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, insulin (10 μg/ml), EGF (20 ng/ml), and penicillin-streptomycin (50 UI/ml and 50 μg/ml, respectively). Isogenic mouse mammary tumor cell lines JDM1.1 (Jak2 WT) and JDM1.2 (Jak2 KO) were derived from a chemically induced mammary tumor (JDM1) of a mouse carrying two conditional knockout (floxed) alleles of *Jak2* (7, 39) that was exposed to DMBA. Primary cells of the parental mammary tumor (JDM1) were infected with the pBabe-puro retrovirus control vector (JDM1.1), or the pBabe-Cre-puro retroviral construct (JDM1.2), which allows a near 100% deletion efficiency of floxed loci after puromycin selection (40). Jak2$^{fl/fl}$ JDM1.1 cells expressed both endogenous Jak1 and Jak2, whereas Jak2-deficient (Jak2$^{-/-}$) JDM1.2 cells expressed Jak1 but lacked the Jak2 protein (Fig. 3B, panels 2 and 4). Cells were maintained in DMEM:F12 media (Life Technologies) supplied with 10% fetal bovine serum, insulin (10 μg/ml), EGF (10 ng/ml), gentamycin (50 μg/ml; Hyclone Laboratories, Logan,

UT), puromycin (7 μg/ml; Hyclone), and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively).

Cell Treatments

For time kinetics and dose responses of Jak1 and Jak2 activation in T47D cells, cells were serum starved for 16–20 h before treatment with 100 nM hPRL for varying times up to 20 min or with varying concentrations of hPRL up to 100 nM for 15 min at 37 C. For Jak1 shRNA experiments (Fig. 6, D and E), cells were treated with 100 nM hPRL for 10 min at 37 C before lysing. All other PRL treatments were performed with 20 nM hPRL for 15 min at 37 C. For adenoviral gene transfer of WT-Jak2, DN-Jak2, LacZ, or hPRLR-L, cells were infected with virus in serum-free media for 90 min at 37 C. Media were then replaced with 10% serum media for 24 h to allow for expression of viral genes. Cells were serum starved for 16 h before stimulation with or without 20 nM hPRL or 20 nM hOSM. Lentiviral infections were carried out for 16 h at 37 C in 10% serum media supplemented with 6 mg/ml Polybrene (Sigma). Media were then replaced with fresh 10% serum media. Forty eight hours after the addition of lentivirus, cells were serum starved for 16 h before PRL stimulation. For Jak2 inhibition, cells were treated with increasing concentrations of AG490 for 16 h in serum-free media before treatment with or without hPRL. Transfection of COS-7 cells was performed with FuGENE6 Transfection Reagent (Roche Pharmaceuticals, Nutley, NJ) according to manufacturer's protocol. RNA was isolated 48 h after transfection for subsequent qRT-PCR analysis.

Solubilization of Proteins and Immunoprecipitation

For protein solubilization, cells were harvested in 1 ml of RIPA lysis buffer (0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 0.15 M NaCl, 0.01 M sodium phosphate, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin) for 1 h at 4 C rotating end over end. Insoluble material was pelleted at 13,000 × *g* at 4 C for 30 min. Individual proteins were immunoprecipitated from clarified lysates using indicated polyclonal antibodies, and captured by incubation with Protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4 C, rotating for 30 min, and washed three times in 1 ml lysis buffer. Immunoprecipitated proteins were dissolved in 2× loading buffer containing reducing agent (Invitrogen, Carlsbad, CA), resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Immunoblotting was performed as previously described (66) with antimouse and antirabbit horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Pierce Chemical Co., Rockford, IL) and exposed to Biomax film (Eastman Kodak Co., Rochester, NY).

Assay of Jak Tyrosine Kinase Activity

T47D cells were incubated with or without 10 nM hPRL for 2 min at 25 C to allow limited receptor activation and partial Jak autophosphorylation. Individual Jak immunoprecipitates from cell lysates corresponding to 1×10^8 cells immobilized on Protein A-Sepharose beads were washed six times in kinase buffer containing 50 mM HEPES (pH 7.3), 100 mM NaCl, 200 μM sodium orthovanadate, 0.1% Triton X-100, and protease inhibitors as described earlier (2, 67). Phosphorylation reactions were performed in a final volume of 100 μl of kinase buffer, with 3 mM $MnCl_2$ and 15 μM ATP for 15 min at room temperature. After the incubation, samples were washed three times in ATP-free kinase buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Antiphosphotyrosine immunoblotting was used to detect autophosphorylated Jak protein.

Adenovirus for Expression Studies

Replication-defective human adenovira (Ad5) carrying WT-Jak2, DN-Jak2, or LacZ were described previously (12). Replication-defective human adenovirus (Ad5) carrying hPRLR was generated using the AdEasy vector system (Qbiogene, Carlsbad, CA). Full-length cDNA encoding the long form of hPRLR (gift from Dr. Paul Kelly) was subcloned into the pShuttle-CMV transfer vector and electroporated into BJ5183 *Escherichia coli* to undergo homologous recombination. Bacterial clones containing recombined adenoviral vectors were screened by kanamycin-resistant growth and confirmed by *Pac*I digestion and sequencing for presence of intact hPRLR cDNA. The recombinant virus was packaged in QBI-293A cells, and resulting adenoviral clones were selected from plaques. Expression of hPRLR from adenoviral stocks was verified by Western blotting using a polyclonal anti-hPRLR antibody (AX901; Advantex BioReagents, Conroe, TX). Selected recombinant viral stocks were expanded in large-scale cultures, purified by double cesium chloride gradient centrifugation, and titered by a standard plaque assay method in QBI-293A cells according to the manufacturer's instructions.

Lentivirus for Delivery of shRNA

pLKO.1-puro lentiviral vectors expressing nontarget control shRNA (SHC002) or one of five different Jak2 shRNAs (TRCN0000003177, TRCN0000003178, TRCN0000003179, TRCN0000003180, TRCN0000003181) or one of four different Jak1 shRNAs (TRCN0000003102, TRCN0000003103, TRCN0000003104, TRCN0000003105) were purchased from Sigma. The lentiviral packaging plasmid pCMV-dR8.2 dvpr (Addgene plasmid 8455) and envelope plasmid pCMV-VSV-G (Addgene plasmid 8454) were kindly provided by Dr. Todd Waldmann (Georgetown University, Washington, DC). 293FT cells (Invitrogen) were cotransfected with shRNA lentiviral plasmid along with pCMV-dR8.2 dvpr and pCMV-VSV-G at a 10:1 ratio for the production of lentiviral particles. Transfections were carried out using Lipofectamine 2000 (Invitrogen), and virus was harvested 72 h after transfection.

RNA Isolation and qRT-PCR Analysis

RNA was isolated from COS-7 or T47D cells using the RNeasy Kit (QIAGEN Inc., Valencia, CA). cDNA was synthesized from isolated RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative PCR analysis was performed using the iQ SYBR Green Supermix (Bio-Rad), where DNA synthesis was monitored in real time by the MyiQ PCR Detection System (Bio-Rad). The following primers were used for real-time qPCR. Jak1: forward, 5′-CTCTGACGTCTGGTCTTTTGG-3′; reverse, 5′-GTTGGGCCTATCATTTTCAGGAAC-3′; Jak2: forward, 5′-TGGAGCTTTGGAGTGGTTCTG-3′; reverse, 5′-TGCCAATCATACGCATAAATTCC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-AATCCCATCACCATCTTCCA-3′; reverse, 5′-TGGACTCCACGACGTACTCA-3′. GAPDH mRNA expression served as an internal control for each sample.

Densitometry

Densitometry was performed using Quantity One software (Bio-Rad). Densities were calculated as an average of five measurements per band with background subtraction. In Fig. 2C, densities were plotted as a percent of maximum intensity.

In Fig. 6F, PRL-inducible bands from phosphoprotein blots were quantified, where each bar depicts the strength of PRL-induced signal in the absence of Jak1 (average of shRNA-2 and shRNA-5 lanes) as a percent of the maximum strength of each PRL-induced signal in the presence of Jak1 (average of mock and nontarget control lanes).

Acknowledgments

Received April 4, 2007. Accepted May 29, 2007.

Address all correspondence and requests for reprints to: Hallgeir Rui, Department of Cancer Biology, Thomas Jefferson University, 233 South Tenth Street, BLSB 330, Philadelphia, Pennsylvania 19107. E-mail: Hallgeir.Rui@jefferson.edu.

This work was supported by United States National Institutes of Health Grants R01-DK52013 and R01-CA101841 (to H.R.) and R01-CA83813 (to H.R. and K.U.W.); U.S. Department of Defense Breast Cancer Research Program Predoctoral Traineeship BC050615 (to L.M.N.); and National Cancer Institute Support Grant 1P30CA56036–08 to the Kimmel Cancer Center. Furthermore, this project is funded, in part, under a Commonwealth University Research Enhancement Program grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations, or conclusions.

Disclosure Statement: The authors have nothing to disclose.

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Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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For more information, please contact:

Dr. Andrzej Bartke
Professor of Physiology and Internal Medicine and Distinguished Scholar
Southern Illinois University School of Medicine
P.O. Box 19628
801 North Rutledge, Room 4389
Springfield, IL 62794-9628, USA
abartke@siumed.edu

or

Dr. Richard E. Falvo,
Adjunct Professor
Department of Cell and Molecular Physiology,
Medical Biomolecular Research Building,
103 Mason Farm Road,
School of Medicine,
University of North Carolina at Chapel Hill,
Chapel Hill, NC 27599-7545, USA
rfalvo@med.unc.edu